

## Quantitative, single-step dual measurement of hemoglobin A1c and total hemoglobin in human whole blood using a gold sandwich immunochromatographic assay for personalized medicine

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### ABSTRACT

We describe a gold nanoparticle-based sandwich immunoassay for the dual detection and measurement of hemoglobin A1c (HbA1c) and total hemoglobin in the whole blood (without pretreatment) in a single step for personalized medicine. The optimized antibody-functionalized gold nanoparticles immunoreact simultaneously with HbA1c and total hemoglobin to form a sandwich at distinctive test lines to transduce visible signals. The applicability of this method as a personal management tool was demonstrated by establishing a calibration curve to relate % HbA1c, a useful value for type 2 diabetes management, to the signal ratio of captured HbA1c to all other forms of hemoglobin. The platform showed excellent selectivity (100%) toward HbA1c at distinctive test lines when challenged with HbA0, glycated HbA0 and HbA2. The reproducibility of the measurement was good (6.02%) owing to the dual measurement of HbA1c and total hemoglobin. A blood sample stability test revealed that the quantitative measurement of % HbA1c was consistent and no false-positive results were detected. Also, this method distinguished the blood sample with elevated HbF from the normal samples and the variants. The findings of this study highlight the potential of a lateral flow immunosensor as a simple, inexpensive, consistent, and convenient strategy for the dual measurement of HbA1c and total Hb to provide useful % HbA1c values for better on-site diabetes care.

### 1. Introduction

Hemoglobin A1c (HbA1c) plays a central role in the diagnosis and monitoring of type 2 diabetes. A current report from the WHO estimates that 80% of global type 2 diabetes-related deaths occur in low- and middle-income countries (WHO, 2015) in which the market for HbA1c tests is limited by the scarcity of the resources. In these countries, inexpensive HbA1c monitoring tools for personalized medicine are crucial in the provision of in-time treatments that slow disease progression and minimize devastating health, social, and economic burdens in afflicted individuals. The development of inexpensive, user-friendly methods for HbA1c testing reduces implementation costs, increases public access to HbA1c test, and improves disease management through by monitoring the % HbA1c.

Ion-exchange high-performance liquid chromatography (HPLC)

and affinity chromatography are generally used to perform HbA1c tests, but the long turnaround time and high implementation cost are incompatible with on-site disease management. Although commercially available point-of-care devices are designed to be mobile for on-site use, their implementation costs limit the use in underdeveloped countries.

The demand for relatively inexpensive point-of-care devices for HbA1c measurement in resource-limited regions has driven ongoing attempts to develop more rapid, consistent, easy-to-use, and cost-effective biosensors for HbA1c measurement. Compared with various HbA1c biosensors such as microarrays (Chen et al., 2012), surface plasmon resonance (Liu et al., 2008), and chemiluminescence flow cells (Ahn et al., 2016), electrochemical biosensors are highly favored for HbA1c detection (Xue et al., 2011; Song and Yoon, 2009; Hsieh et al., 2013) because of their compatibility with miniaturization into portable point-of-care devices (Liu et al., 2012). However, the multiple steps of results interpretation and measurement that require professional personnel increase the implementation costs of this method. Such limitations have driven

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the growth of lateral flow assays that use relatively inexpensive materials (e.g., paper) to provide simple, rapid, on-site results.

Although lateral flow assays are commonly designed to perform qualitative detection in which an on/off signal is sufficient (Posthuma-Trumpie et al., 2008), the invention of reflectance readers has made it possible to measure the signal transduced on the paper-based platform, which allows the quantitation of target analytes with lateral flow strips. Aimed at reducing the cost of HbA1c tests to cater to the needs of patients in underdeveloped regions, two lateral flow strip designs for the detection and measurement of HbA1c have been reported (McCroskey and Melton, 2010; Sundrehagen, 2014). Although these designs could cater to the demand for low-cost HbA1c assays, their testing procedures, which involve either a two-step measurement or an elaborate pretreatment of whole blood before testing, still require professional personnel.

In this study, the drawbacks of previously reported HbA1c lateral flow strip designs are addressed with the development of a lateral flow immunosensor that directly detects both total Hb and HbA1c in human whole blood without sample pretreatment. The dual-detection design of the immunosensor substantially simplifies the workflow and allows interpretation and measurement to be performed in a single step. Compared with previously reported lateral flow assays that use biomimetic boronate groups, which can bind to all of the glycan moieties that readily coexist with HbA1c in whole blood, sandwich immunoassays are reportedly more selective and sensitive in probes for multi-epitope antigens such as Hb (Ngom et al., 2010). In addition to being more selective than the previously reported lateral flow assays, when compared to platforms that measure only absolute % HbA1c (Chuang et al., 2012), this lateral flow immunosensor that measures both HbA1c and total Hb not only allows more stable estimation of % HbA1c by compensating for intra- and inter-variation in total Hb but is also particularly useful in patients with sickle cell anemia, in which the amount of Hb is decreased. For these patients, the dual detection of HbA1c and total Hb is helpful in distinguishing true-negative from false-negative results because the HbA1c levels can be falsely low when only absolute % HbA1c is measured. The successful incorporation of a dual-detection technology in a simple, user-friendly, and inexpensive platform promotes efforts to improve the accessibility of HbA1c tests, particularly in remote settings.

## 2. Materials and methods

### 2.1. Chemicals and materials

Naked gold nanoparticles (40 nm) were purchased from Kestrel Biosciences Co., Ltd. (Pathumthani, Thailand), and bovine serum albumin was purchased from Amresco LLC (Solon, OH, USA). Western blocking reagent (10%) was obtained from Roche Diagnostics (Selangor, Malaysia). Anti-HbA1c antibody (monoclonal, IgG1), anti-Hb antibody (polyclonal, goat), rabbit anti-goat polyclonal antibody, purified HbA1c, HbA0, glycated HbA0, and HbA2 were purchased from Fitzgerald Industries International (Acton, MA, USA). Hemolysis reagent was purchased from Kamiya Biomedical Company (Seattle, WA, USA). All other chemicals were purchased from Sigma-Aldrich (Selangor, Malaysia). Phosphate buffer (pH 7.4) was prepared with  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ , and NaCl,  $\text{K}_2\text{CO}_3$ , and HCl were prepared with Milli-Q water with a resistivity of 18.2 M $\Omega$  cm. Antibodies and purified HbA1c, HbA0, and glycated species of HbA0 and HbA2 were diluted with phosphate buffer. Laminated nitrocellulose (NC) membrane cards (ref. HF135MC100), cellulose fiber pads (ref. CFSP173000), and glass fiber pads (ref. GFPO83000) were acquired from Merck Millipore

(Selangor, Malaysia). Blood samples were collected from the University of Malaya Medical Center (UMMC), and informed consent was obtained from the patients recruited. The study protocol was implemented in accordance with the policies of the institutional medical ethics board committee.

### 2.2. Apparatus

Test strips were cut by using a Matric 2360 programmable shear from Kinematic Automation (Sonoma, CA, USA). An Infinite® M200 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland) was used to measure the absorbance of the gold conjugates (at 530 nm). An ESEQuant lateral flow reader (Qiagen Lake Constance GmbH, Stockach, Germany) was used for quantitative measurement of the signal on the immunosensor.

### 2.3. Conjugation of detecting antibody to gold nanoparticles

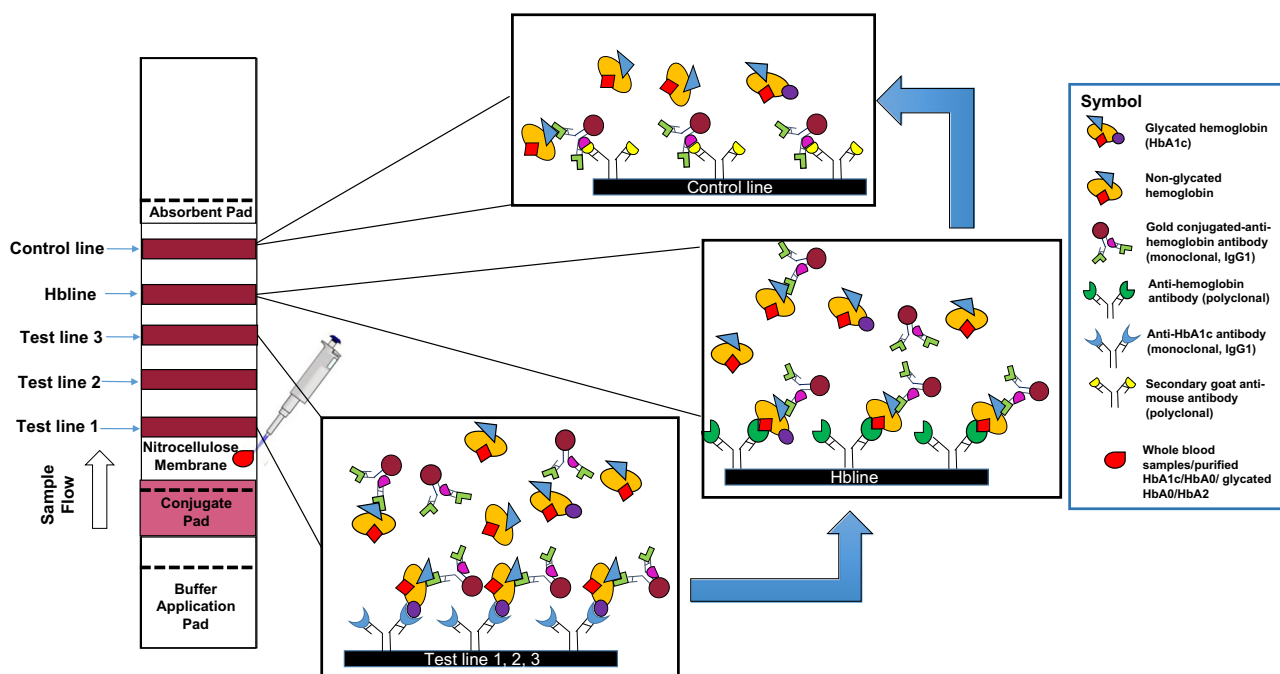
Gold conjugates were prepared with 10 mL colloidal gold nanoparticles adjusted to pH 8.0 and polyclonal anti-Hb antibody at a final concentration of 30.0  $\mu\text{g mL}^{-1}$ . The gold conjugates were incubated for 1 h with gentle shaking (100 rpm) at room temperature. Next, bovine serum albumin at a final concentration of 1% (w/v) was added to the gold conjugates and allowed to shake for an additional hour. The gold conjugates were centrifuged at 11627g for 30 min at 4 °C to yield a soft pellet, which was then resuspended in 10 mM phosphate buffer containing 1% (w/v) bovine serum albumin. The conjugates were stored at 4 °C until use.

### 2.4. Construction of the HbA1c lateral flow immunosensor

The lateral flow immunosensor was composed of a buffer application pad, conjugate pad, laminated NC membrane, and absorbent pad (Scheme 1) and comprised five lines: three HbA1c test lines coated with anti-HbA1c antibody (monoclonal, IgG1), one Hb line on which anti-Hb antibody (polyclonal, goat) was immobilized, and one control line coated with secondary polyclonal rabbit anti-goat antibody. The antibodies were lined manually with a pipette. After lining, the strips were dried in a desiccator for 30 min. Later, they were blocked with 1% (w/v) western blocking reagent in 10 mM phosphate buffer. The gold-conjugated polyclonal anti-Hb antibody (detecting antibody) prepared in 10 mM phosphate buffer supplemented with 10% (w/v) sucrose were deposited onto the glass fiber conjugate pad to dry overnight in a desiccator. The full lateral flow immunosensor was then assembled with the cut buffer application pad, dried conjugate pad, and laminated NC membrane with an absorbent pad (4-mm width). Overlap between the conjugate pad and NC membrane was optimized at 1 mm, whereas the NC membrane and absorbent pad overlapped at 2 mm.

### 2.5. Optimization of the detection format for the sandwich immunoassay

To develop a functional sandwich immunoassay that could detect total Hb and HbA1c simultaneously, we used two formats: one with the Hb line first (closest to the conjugate pad) and one with the Hb line toward the end of the strip (closest to the control line). The two formats were tested with three blood samples with different % HbA1c values (5.3%, 6.5%, and 9.0% HbA1c, diluted 1:5 with hemolysis reagent). After depositing the diluted blood onto the NC membrane, we added washing buffer to complete the assay. Patterns were then observed to determine the ideal detection format for the sandwich immunoassay. The results from quantitative analysis (determined by an ESEQuant lateral flow reader) for these two formats were presented in Table S1, Supplementary



**Scheme 1.** Schematic representation of simultaneous detection of HbA1c and hemoglobin using sandwich immunoassay on lateral flow strip.

materials.

## 2.6. Selectivity test

The assembled HbA1c immunosensor was tested with purified HbA0, HbA1c, glycated HbA0 and HbA2. All purified reagents were prepared in 10 mM phosphate buffer to a final concentration of  $0.1 \text{ mg mL}^{-1}$ . The immunosensor was tested in triplicate with  $10 \mu\text{L}$  diluted purified antigen. After the purified antigen was dispensed, the assay was completed with the addition of washing buffer (50 mM phosphate buffer containing Tween-20) and the signal generated on the strips was measured with the ESEQuant lateral flow reader.

## 2.7. Investigation of the interference by variants

Three variant blood samples, HbE, HbJ, and HbH, were collected with the anticoagulant ethylenediaminetetraacetic acid and verified with a Bio-Rad Variant II HPLC (Hercules, CA, USA). These samples were collected from the hematology lab and tested on the seventh day after withdrawal from patients or donors. A blood sample with elevated HbF was discovered incidentally during the study and was tested on the same day it was withdrawn from the patient. All four samples (three variants and the elevated HbF sample) were diluted 1:5 with hemolysis reagent before testing. The signals were measured with the lateral flow reader.

## 2.8. Determination of the effects of long storage on the analysis

Over 7 days, one blood sample with 6% HbA1c was tested on the fabricated strips. Throughout the study, the sample was diluted 1:5 with hemolysis reagent. The diluted sample was dispensed directly onto the NC membrane. Later, the strips (triplicates for each point of testing) were washed with 50 mM phosphate buffer containing Tween-20. The signals generated on the strips were measured with the lateral flow reader. A two-tailed *t*-test was performed to determine the significance of the difference in signals observed over 7 days.

## 2.9. Reproducibility study

Nine lateral flow immunosensors were tested with a diluted blood sample of 5.0% HbA1c (determined with the Bio-Rad Variant II HPLC). Blood samples were diluted 1:5 with hemolysis reagent (distilled water with blood stabilizers) before testing, and washing buffer was added to complete the assay. Signals were measured with the ESEQuant lateral flow reader.

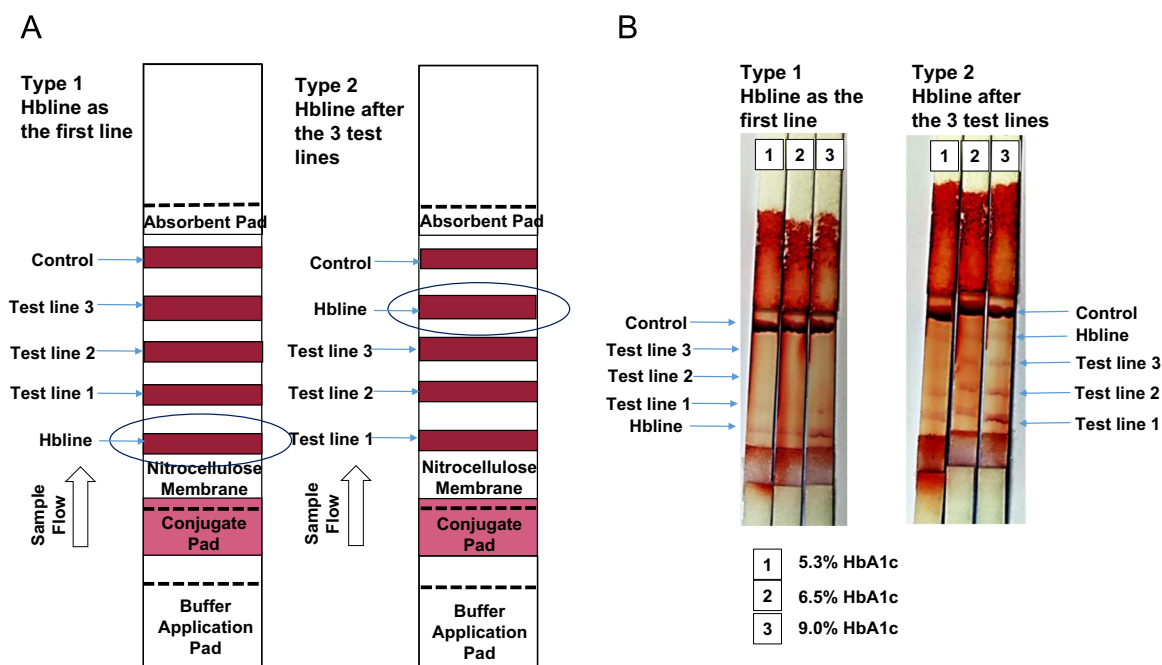
## 2.10. Generation of the calibration curve

The calibration curve was generated based on the HbA1c/total Hb ratio as a function of % HbA1c. To factor in all pre-existing interfering agents in whole blood, we established the calibration curve with whole blood samples collected from UMMC. The signal intensities for the test and Hb lines were measured for each % HbA1c sample collected and the ratio was calculated. All whole blood samples were diluted with the hemolysis reagent before testing. The signals generated were measured with the ESEQuant lateral flow reader.

# 3. Results and discussion

## 3.1. Dual-detection strategy

**Scheme 1** is a schematic illustration of single-step dual detection of HbA1c and total Hb on the same sensing platform. This lateral flow test strip was designed with an Hb line, three test lines for the specific detection of HbA1c, and a control line (five lines total) for simultaneous detection of HbA1c and total Hb. Antibody-functionalized gold nanoparticles were optimized to perform sandwich immunoreaction at test lines 1–3 and the Hb line, the results of which were transduced into a visible signal on the strip. The transduced signal was read semi-quantitatively with the naked eye and measured quantitatively with a lateral flow reader. With the transduced immunoreactions occurring at distinctive lines after detecting HbA1c and Hb, the reported lateral flow immunosensor provided the ratio of HbA1c to total Hb, which is particularly useful for the measurement of % HbA1c.



**Fig. 1.** (A) Designs of the Type 1 and Type 2 detection format, with the Hb line positioned either before or after the three HbA1c test lines. (B) Whole blood testing with increasing % HbA1c using the type 1 and type 2 design.

### 3.2. Optimization of the detection format of the sandwich immunoassay

Fig. 1a illustrates the two types of detection formats examined. The position of the Hb line either before or after the three test lines significantly affected the pattern observed on the strips. The Type 1 design, with the Hb line placed before the three HbA1c test lines, showed an increased intensity at the Hb line as the % HbA1c in blood samples increased (Fig. 1b). However, the test lines coated with anti-HbA1c antibodies (test lines 1–3) were too pale to be observed. We therefore presumed that when the Hb line was set as the first line, the assay captured most of the Hb in the blood samples, including HbA1c. Because most of the HbA1c was captured at the first Hb line, only a negligible amount passed through the immunocomplexes formed on the Hb line and reached the subsequent HbA1c test lines to immunoreact with anti-HbA1c antibodies (monoclonal, IgG1).

However, with the Type 2 design, in which the Hb line was placed after the three HbA1c test lines, we observed an increase in the intensity and number of HbA1c test lines that appeared concomitantly with the increase in % HbA1c. The consequent saturation of the binding sites at the first test line allowed unbound HbA1c to migrate to subsequent lines for further immunoreaction. All of the unreacted Hb species were flushed to the last line – the Hb line – to bind with the polyclonal anti-Hb antibodies. The results of our detection format optimization studies showed that with the type 2 design, the intensity gaps were sufficiently large with increasing % HbA1c and allowed monitoring for distinct HbA1c ranges (normal, < 6.5% HbA1c; under control, 6.5% to 7.0% HbA1c; elevated, > 7.0% HbA1c). Hence, we chose the type 2 format for the simultaneous detection of HbA1c and total Hb.

### 3.3. Selectivity study

Because the polyclonal anti-Hb antibody (detecting antibody) – functionalized colloidal gold binds to all Hb species (glycated or non-glycated), it was critical to ensure that the anti-HbA1c antibodies recognized only the glycation site specific to HbA1c (valine at the N-terminal of  $\beta$  subunits) and transduced signal only from

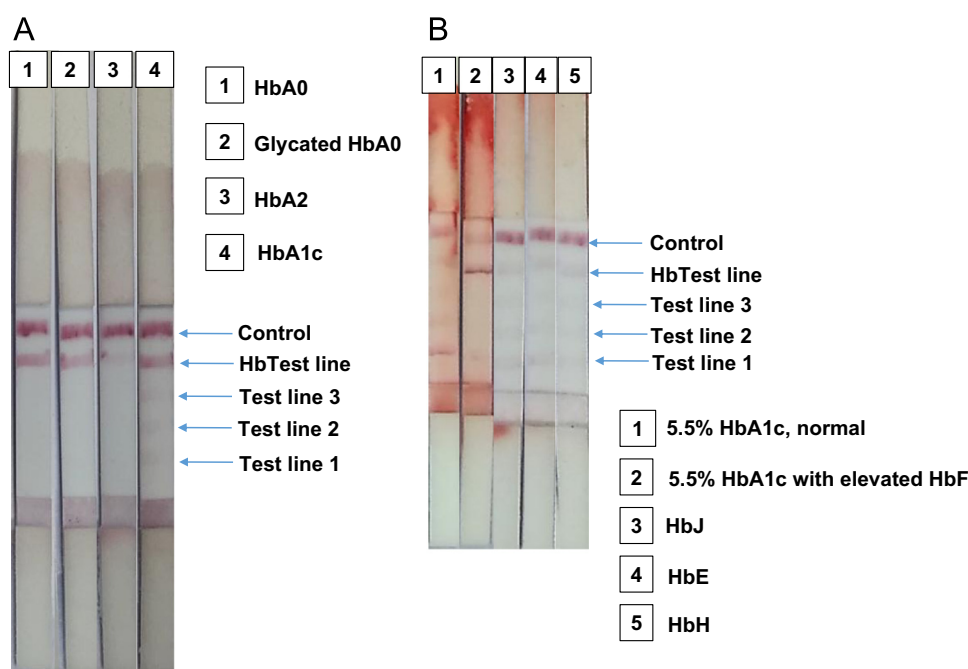
bound HbA1c. The HbA0 species contains the same  $\alpha\beta$  subunits found in HbA1c (Ang et al., 2015), in which similar glycan moieties are highly likely to be found; therefore, they could potentially interfere with the results generated on the strip. HbA2, which contains  $\alpha\delta$  subunits, might have glycan moieties on the  $\alpha$  chains, which could interfere with the signal generated if the capturing anti-HbA1c antibodies are not specific enough.

In the selectivity studies shown in Fig. 2a, only purified HbA1c showed responses on test lines 1–3 (on which monoclonal anti-HbA1c antibodies were coated). HbA0 and glycated HbA0 and HbA2 yielded no results on test lines 1–3. These observations led us to conclude that the design of the sandwich immunoassay was highly selective for HbA1c. Other glycation sites on Hb were not recognized, and a “sandwich” was not formed even upon binding and forming immunocomplexes with the polyclonal anti-Hb antibody-functionalized colloidal gold.

### 3.4. Interference of variants

Fig. 2b shows that at the same HbA1c level (5.5% HbA1c, strip 1), the Hb line on strip 2 (tested with a sample that also contained elevated HbF) was relatively more intense (483% higher), and test lines 1–3 were comparatively paler (80.2% paler) than those on strip 1. These observations agreed with those reported by Rohlfing et al. (2008) for DCA 2000 (which uses an immunoturbidimetric method) in which the presence of elevated HbF artificially lowered the % HbA1c. HbF comprises  $\alpha\gamma$  subunits in which the glycine at the  $\gamma$  subunit is more readily acetylated than glycated (Rohlfing et al., 2008), which suggests that the observation of lower intensity on test lines 1–3 was expected, particularly when the monoclonal anti-HbA1c antibodies used were proven to be highly selective toward the glycation sites at valine in the N-terminus of the  $\beta$  subunit. Whereas elevated HbF artificially lowered the HbA1c level on the strip, the population of HbA constituted a relatively larger portion of the whole Hb population in the sample, which contributed to the high intensity at the Hb line.

We tested three additional variants – HbH, HbE, and HbJ – with the immunosensor. When HbJ was tested on strip 3, test lines 1–3 remained visible, but the intensity of the Hb line was lower than



**Fig. 2.** (A) Four purified cross-reactants were tested on the fabricated immunosensor. (B) Variant interference study conducted with a sample of elevated HbF, HbJ, HbE, and HbH.

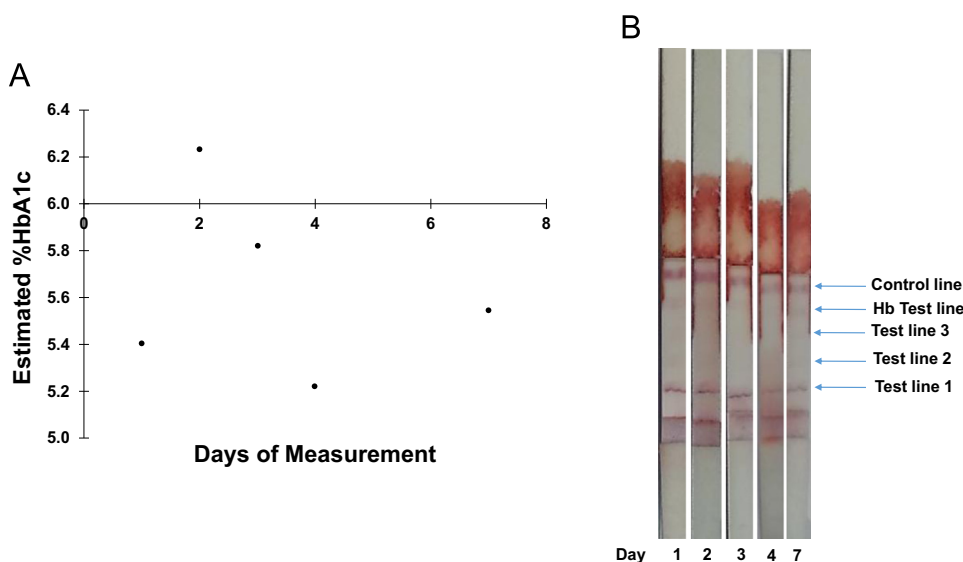
that on strip 2 (tested with elevated HbF). HbJ is a variant that, similar to HbA, contains  $\alpha\beta$  chains with a mutation that adds a negative charge to the Hb molecules, thereby affecting the accuracy of the cation-exchange method and resulting in artificially low HbA1c values (Bhat et al., 2012), (Tsai et al., 2001). Our immunosensor, which uses highly selective monoclonal anti-HbA1c antibodies, was partially affected by the presence of HbJ and gave a falsely low HbA1c level.

On the contrary, compared with strips 3 (tested with HbJ) and 5 (tested with HbH), strip 4 (tested with HbE) showed a relatively similar intensity on the Hb line. The intensities of test lines 1–3 were considerably lower than that observed on strip 1. HbE has a mutation farther away from the N-terminal of the  $\beta$  chain (Little and Roberts, 2009) that could cause a degree of structural incongruence with anti-HbA1c antibodies. Similar observations were

made for strip 5, which was tested with HbH. HbH has a mutation on the  $\alpha$  subunit. The HbA1c level measured in ratio was falsely low and agreed with the findings of Pravatmuang et al. (2001), who presumed that the presence of the HbH variant lowered the HbA1c level because the polymerized  $\beta_4$  eluted as a non-quantitating area with the HPLC method. However, we hypothesized that the polymerization of  $\beta_4$  leads to structural changes that make the glycated site on the  $\beta$  chain more difficult to probe. All three variants interfered with HbA1c measurement and resulted in falsely low HbA1c measurements.

### 3.5. Blood specimen storage stability study

Fig. 3 shows that consistent signals were generated on the strips when the same 6% HbA1c blood sample was tested over



**Fig. 3.** (A) The estimated %HbA1c versus days of measurement. (B) No significant difference (Table 1, supporting information) was found between the signals generated on the first day of collection versus the signal generated at every point of the testing day.



7 days. To investigate the significance of the signal intensity differences observed during the study, we performed a two-tailed *t*-test comparing the results at each point of the testing to that on day 1. No significant difference (all *t* statistical values fell within the range of the  $\pm t$  critical two tail) was observed in the comparison of HbA1c measurements on day 1 with those on each testing day for 7 days (Table S2a–d). Although the signal intensity on later days was comparatively lower (Fig. 3b) than that on the first day of testing, the ratio of HbA1c to total Hb remained constant. From the ratio obtained, we estimated % HbA1c by using the calibration curve established, in which the % HbA1c was found to lie within 5.2–6.2% HbA1c compared with the true value (6% HbA1c; Fig. 3a). After storing the sample for 7 days, measurement of % HbA1c provided a good estimation, and no false-positive results ( $\geq 6.5\%$  HbA1c) were detected.

These observations showed that the blood sample remained stable at 4 °C, and storage had little to no effect on the measurement of % HbA1c if the test was conducted within 7 days of storage at that temperature. Unlike Lakshmy and Gupta (2009), who reported that HbA1c is stable for up to 15 days in dried blood samples, and Rohlfing et al. (2012), who reported a 14-day window for HbA1c measurement when samples are stored at 4 °C (Rohlfing et al., 2012), we reported on 7 days of storage at 4 °C because it is common practice for blood samples to be kept for that duration at UMMC before they are discarded.

### 3.6. Reproducibility

The coefficient of variation (CV) of the lateral flow immunosensor was found to be 6.02% in the reproducibility test (Fig. S1). By relating the CV obtained to the physiological variance (potential HbA1c level difference every time a measurement is performed on the same sample), it was calculated to be 1.5% HbA1c difference in physiological context (please refer to the detailed calculation made for physiological variance in the Supplementary materials). With the physiological variance measured to be 1.5% HbA1c, the signals generated on the test strip with the same HbA1c level are expected to be consistent and precise. On the other hand, to relate the difference of ratiometric measurement to physiological context, the calibration curve relationship obtained  $Y = 0.0556X + 0.4491$  (Fig. 4) was utilized. For example, the difference of 0.02 in ratiometric measurement would result in a 0.2% HbA1c difference, so as 0.18–3.2% HbA1c difference (detailed calculation made was presented in the Supplementary materials), indicating that a small change in ratiometric reading could result in a significant difference of % HbA1c in physiological context. In other words, our device is reasonably sensitive, accurate and precise in relating the ratiometric reading to physiological change in HbA1c level and therefore its utility for personalized medicine is justified.

Compared with the reported CVs (0.19–0.82%) of other biosensors such as immunoassay microarrays (Chen et al., 2012), the CV of our assay was higher. Nevertheless, tests with our lateral flow immunosensor can be completed more rapidly (20 min) than those using microarray immunoassays (which require 2 h of incubation), and is therefore more practical for on-site facilitation of diabetic care. Compared with reported potentiometric methods (Liu and Crooks, 2012), our lateral flow immunosensor not only requires less sample volume for testing (2  $\mu$ L) but also has high selectivity for HbA1c detection – without any sample pretreatment. Compared with the separation matrix, our lateral flow strip requires no multistep measurement to obtain a signal for both HbA1c and total Hb. Owing to the optimized design for simultaneous detection of both HbA1c and total Hb, signal measurement can be performed in a single step with one test run. Table 1 shows a comparison of the overall analytical performance of the

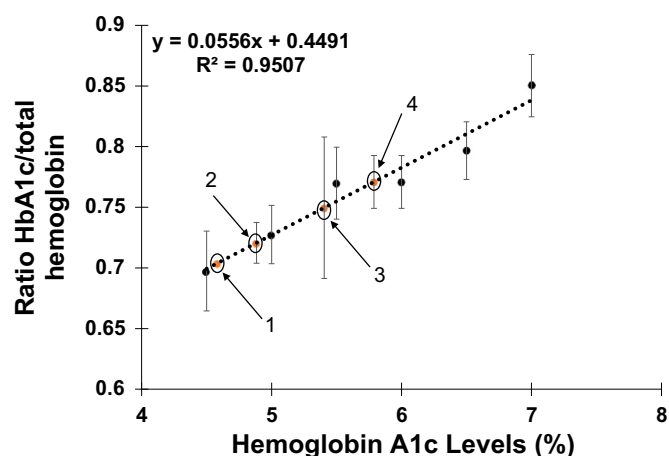


Fig. 4. HbA1c/total hemoglobin ratio versus % Hemoglobin A1c. Error bars indicated standard deviations with  $n=3$ . Unknown samples 1, 2, 3 and 4 were circled to show the estimated HbA1c level using the calibration curve. The estimated HbA1c levels for samples 1, 2, 3, and 4 were compared to Bio-Rad Variant II HPLC method.

developed lateral flow immunosensor and other biosensors.

### 3.7. Calibration curve

Fig. 4 shows that the linear dynamic range established with whole blood fell within 4.5% (26 mmol mol<sup>-1</sup>) to 7% (53 mmol mol<sup>-1</sup>) HbA1c, with a  $R^2$  of 0.95. This result indicates a strong linear relationship between % HbA1c and the signal ratio (HbA1c/total Hb) generated on the immunosensor. The small value of the slope established with the calibration curve suggested that the changes in HbA1c levels were subtle compared with the consistent and constant total Hb concentration. Although the dynamic range of detection was narrow, the critical treatment goal values of 6.5–7% HbA1c falling within the linear dynamic range (4.5–7.0% HbA1c) rendered the developed lateral flow immunosensor a useful device for diabetes care, especially for patients with poorly controlled glycemic status with HbA1c levels of  $> 6.5\%$  (Group, 2005) (or  $> 7\%$  in the United States) (ADA, 2012).

Points marked 1, 2, 3, and 4 in Fig. 4 indicate responses obtained for unknown whole blood samples. Using the calibration plot, we estimated the HbA1c level in the unknown blood samples to be 4.6%, 4.8%, and 5.4%, and 5.8% HbA1c, whereas the Bio-Rad Variant II HPLC measured HbA1c levels of 4.5%, 4.7%, and 6.0%, and 6.0% for sample 1, 2, 3, and 4, respectively (note: sample 3 and 4 were two different samples but both having same HbA1c level). A two-tailed *t*-test for the set of measurements gave a *p* value of 0.79 and a *t* statistical value of  $-0.28$  that fell within  $\pm 2.57$ , which led us to conclude that there was no significant difference in the HbA1c levels measured with the Bio-Rad Variant II HPLC and our immunosensor. However, when the estimated 5.4% HbA1c level of unknown sample 3 ( $N=3$ ) was compared to HPLC-measured value of 6.0% HbA1c, the 0.6% difference in measurement could be a concern when it comes to diagnosing diabetes. We believe that with further experimentation on larger sample size ( $N \geq 30$ ), outliers like unknown sample 3 could be addressed and so to improve and tighten the variance between our assay and HPLC method to a clinically relevant variance of 3–5% (Sacks et al., 2011).

## 4. Conclusions

Herein we report a robust and promising lateral flow HbA1c immunosensor designed to allow HbA1c to be measured as a

**Table 1**

Comparison on the analytical performances of our developed HbA1c lateral flow immunosensor versus other HbA1c biosensors.

	HbA1c lateral flow immunosensor (this study)	Microarray immunoassay (Chen et al., 2012)	Potentiometric method (Liu and Crooks, 2012)	Separation matrix (McCroskey and Melton, 2010)
Bio-recognition material	Antibodies	Antibodies	Boronic acid	Boronic acid
Sample volume required	2 $\mu$ L	4 $\mu$ L	50 $\mu$ L	2–5 $\mu$ L
Clinical testing	Yes	Yes	No	Yes
Pretreatment	No	No	Yes	No
Rapidity of test	Approximately 20 min	2 hour incubation prior to test	Approximately 20 min	Approximately 5 min
Sensitivity	NA <sup>a</sup>	0.20 ng mL <sup>-1</sup>	NA	NA
Reproducibility	6.02%	0.19–0.82%	NA	1.7%
Linear dynamic range	4.0–7.0% HbA1c	NA	NA	5–16% HbA1c

<sup>a</sup> NA – Not available in the study.

fraction of total Hb with a single test run that involves only washings and single-step measurement. Because HbA1c is measured as a fraction of total Hb, we compensated for the reduction in the immunoreaction between anti-Hb antibodies and Hb by using the parallel reduction in HbA1c binding for stable measurement of the signal ratio to calculate % HbA1c.

Good consistency was demonstrated without false-positives when blood samples stored at 4 °C for a week were tested. Furthermore, the anti-HbA1c antibodies coated at the test lines showed high specificity and selectivity toward HbA1c when tested against all other glycosylated proteins (HbA0 and glycosylated HbA0 and HbA2). Moreover, we were able to distinguish samples with normal Hb from those with elevated HbF owing to high intensity at the Hb line on the strip. However, the presence of Hb variants (HbJ, HbE, and HbH) and elevated HbF affected the measurement of % HbA1c. Therefore, we suggest pre-screening for the presence of variants before performing the definitive test.

With the constructed calibration curve, quantitative measurements of % HbA1c did not differ significantly between the lateral flow immunosensor and the Bio-Rad Variant II HPLC. To improve and expand the dynamic range of detection of % HbA1c, we will optimize the reaction matrix of our lateral flow strips in future studies by using different types of membranes. We anticipate that our immunosensor, developed to integrate inexpensive detection technologies such as smartphone-based imaging, will enable rapid point-of-care evaluation of HbA1c levels in blood samples to improve on-site diabetes care.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2015.11.045>.

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